Extraction of taxol, an anticancer drug from coelomycetous fungi *Pestalotiopsis versicolor* and *Phyllosticta murrayicola*

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Abstract: Two different coelomycetous fungi were screened for the production of taxol, an anticancer drug. Taxol production was confirmed by the following methods: Ultra Violet (UV) spectroscopic analysis, Infra Red analysis (IR), High performance liquid chromatography analysis (HPLC) and Liquid chromatography mass spectrum (LC-MASS), and the taxol compared with authentic taxol. The fungal taxol was identical to authentic taxol. The taxol produced by the above fungi was tested against cancer A549 cell line.

Key words: A549 cell line, *Pestalotiopsis versicolor, Phyllosticta murrayicola,* taxol production

Introduction

The importance of natural products to drugs discovery, in particular, anticancer drug has historically been very large (Stierle *et al.* 1993). Natural products have provided some of the most significant cancer chemotherapeutics (Kingston 1994), largely because they provide drugs that are inaccessible by other routes. Compounds such as paclitaxel (Taxol) have never been prepared by standard medicinal chemistry approaches to drug discovery. Isolation of bioactive natural products offers a unique and effective route to the discovery of new anticancer agents (Pinkerton & Strobel 1976). The process is not easy, however, and successful discovery of a new drug substance requires an excellent collection of natural extracts, a selective and predictive bioassay, and skilled isolations chemists (Schiff & Horowitz 1980).

Taxol, a diterpene was originally isolated from the bark of Pacific Yew tree (*Taxus brevifolia*) more than two decades ago and has proved to possess anticancer activity. The US National Cancer Institute, in collaboration with Bristol Myers Squibb Co and other workers have demonstrated the efficacy of taxol against certain human cancer. Its mode of action is unique in that it inhibits mitosis through enhancement of polymerization of tublin and consequent stabilization of microtubles during the process of cell division. However, a complete treatment for the patient requires 2 g of taxol, administered several times a day and for many months. To obtain 1 kg of taxol requires about 10 000 kg of bark (Vidensik *et al.* 1990), and several thousand trees must be cut to procure this quantity of bark. This scarcity of taxol and the ecological impact of harvesting it encouraged scientists to find alternative methods using microorganisms (Stierle *et al.* 1993; Strobel *et al.* 1997). A coelomycetous fungus, *Pestalotiopsis microspora,* an endophyte from inner bark of *Taxus wallachiana* produced taxol in culture. Keeping this in mind, an attempt has been made to examine the production of taxol by some other coelomycetous fungus as well. The taxol isolated from these fungi is biologically active against cancer cell lines. In order to reduce the price of taxol and make it, available in higher quantities a fermentation process involving microorganisms would be the most desirable means of supply. It was first discovered by Strobel *et al.* (1996a), that the fungus *Taxomyces andreanae* could produce taxol, through the yield was low. Strobel *et al.* (1996b) and Wang *et al.* (2000) showed that *Pestalotiopsis microspora* isolated from the bark of *Taxus wallachiana* produced taxol in mycelial culture. This work prompted us to continue the search for taxol production from fungal sources.

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Material and methods

Plant material and experimental methods

Fresh leaves of *Acacia meanoxylon* and *Murraya koenigii* were carefully washed in sterile distilled water and dried. The samples were placed on potato agar dextrose plate and after 72 h the mycelium produced were isolated and sub-cultured (Booth 1971).

Taxol extraction

Extraction of taxol was performed according to Strobel *et al.* (1994). After incubating the culture for 3–4 weeks, the culture filtrate was passed through four-layered cheesecloth. In order to avoid fatty acid contamination of taxol, 0.25 g of $NaCO_3$ was added to the filtrate. The culture fluid was extracted with two equal volumes of methylene chloride and the organic phase was evaporated to dryness under reduced pressure at 35 °C.

Column chromatography

A 1.5 × 30 cm column of silica gel was loaded with crude sample dissolved in methylene chloride. Elution of the sample was done in a stepwise manner with solvent system as 70 ml of 100 % methylene chloride, 20:1 v/v methylene chloride : ethylacetate, 10:1 v/v methylene chloride : ethylacetate, 6:1 v/v methylene chloride : ethylacetate, 3:1 v/v methylene chloride : ethylacetate and 1:1 v/v methylene chloride : ethylacetate. Fractions having same mobility as that of authentic taxol were combined and evaporated to dryness. The residue was subjected to thin layer chromatography.

Thin layer chromatography

TLC analysis was carried out on Merck 1 mm (20 × 20 cm) silica gel plate was carefully removed by scraping off the silica at the appropriate Rf and eluted with acetonitrile. Taxol was detected with 1 % w/v vanillin / sulphuric acid reagent after gentle heating (Cardellina 1991). It appeared as a bluish spot that faded to dark grey after 24 h.

Ultra violet spectroscopy analysis

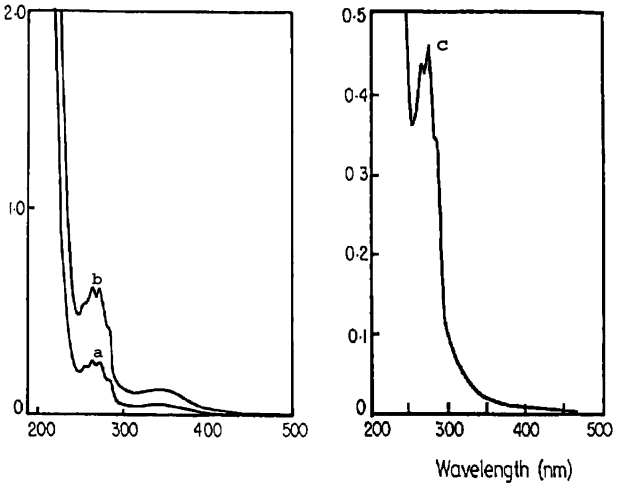
The purified sample of taxol was analyzed by UV absorption, dissolved in 100 % methanol at 273 λ_{max} and compared with authentic taxol (Wani *et al.* 1971).

Infra red spectroscopy analysis

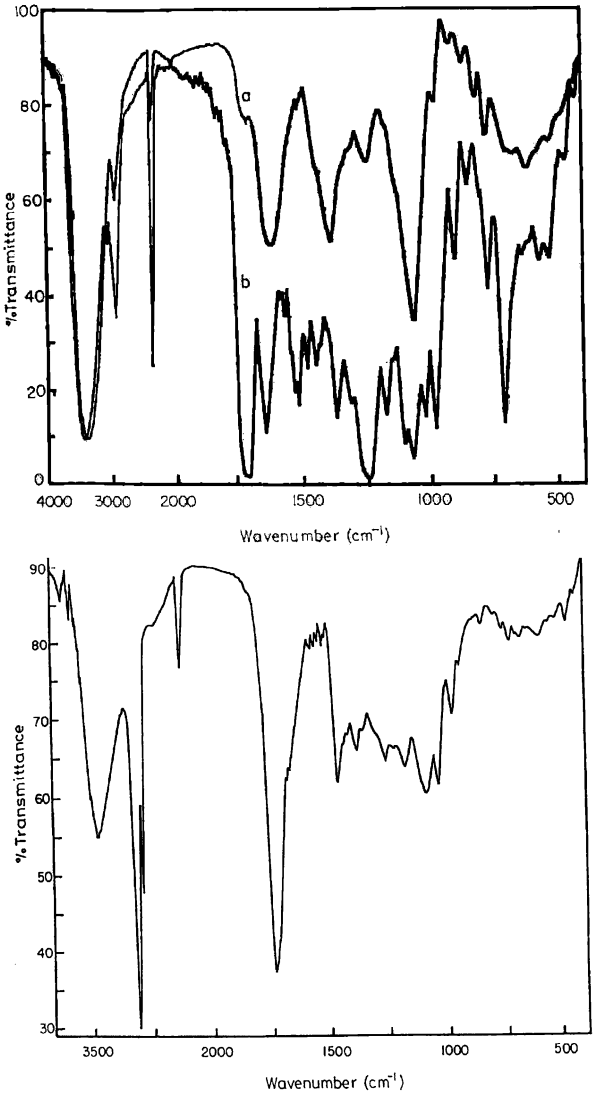
The purified taxol was ground with IR quality potassium bromide (1:10) pressed into discs under vacuum using spectra lab Pelletiser and the Spectrum was recorded (4000–500 cm^{-1} nm) in a Burker 17S 85 FTIR Spectrophotometer.

High performance liquid chromatography

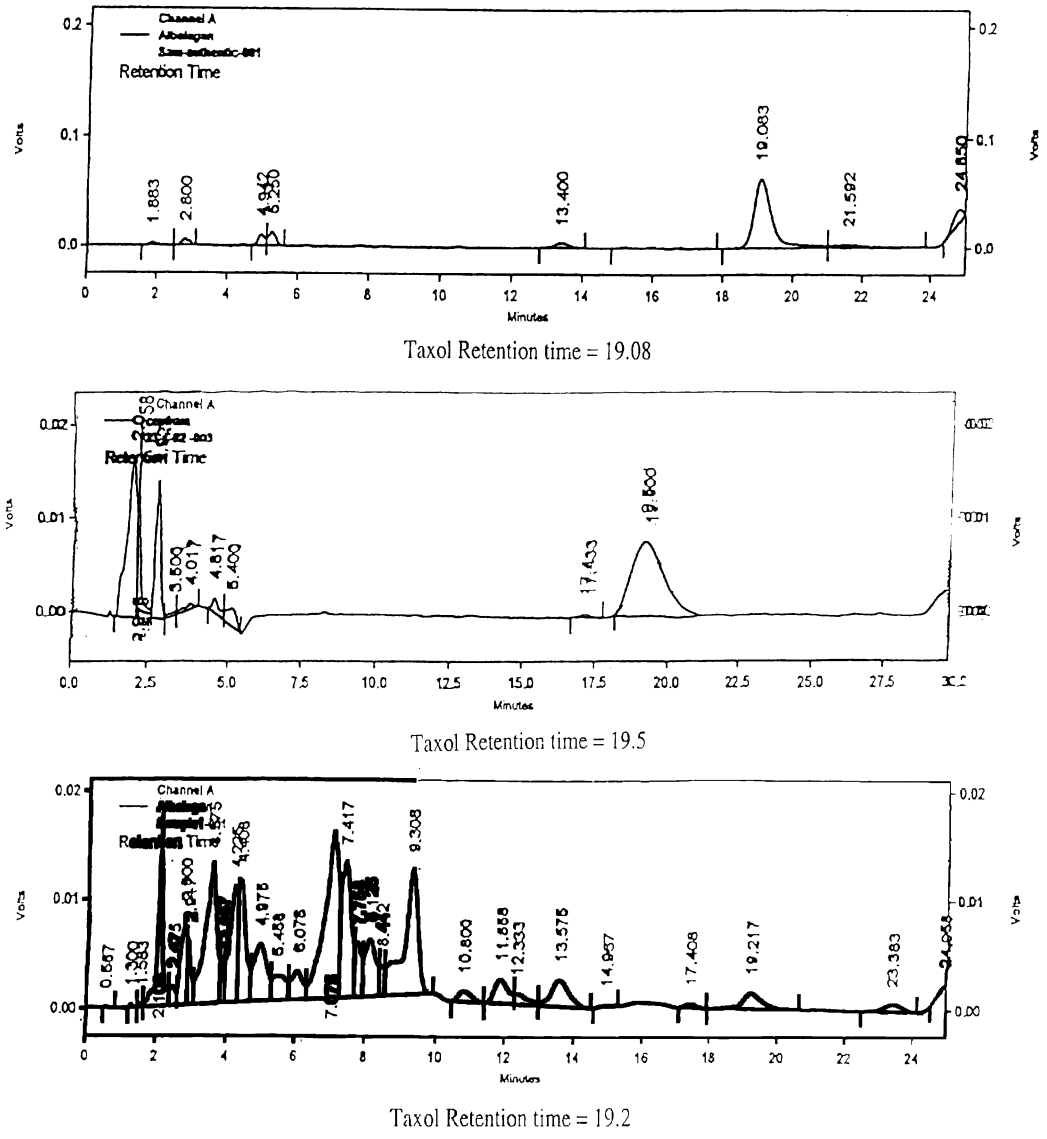
To further confirm the presence of taxol in the fungal extracts, the culture filtrate of the fungus was extracted with chloroform: methanol, 7:1 v/v and subjected to TLC with the solvent system chloroform : acetonitrile, 7:3 v/v. The putative taxol was scraped and eluted with acetonitrile. The resulting



Figs 1a–c. Ultra Violet absorption spectra of (a) authentic taxol, (b) *Pestalotiopsis versicolor,* (c) *Phyllosticta murrayicola*



Figs 2a–c. IR spectrum of (a) authentic taxol, (b) *Pestalotiopsis versicolor,* (c) *Phyllosticta murrayicola*



Figs 3a–c. HPLC analysis of (a) authentic taxol, (b) *Pestalotiopsis versicolor,* (c) *Phyllosticta murrayicola*

residue was subjected to micropore HPLC separation for optimum sensitivity. The study was conducted on a reverse phase 1 mm × 150 mm × 5 mm particles with isocratic mobile phase consisting methanol : water, 80:20 v/v at the flow rate of 50 µl/min. Subsequent analysis of 2 µl of the sample prepared from 100 µl of the dissolved sample was conducted.

Liquid chromatography mass spectrum analysis

Samples selected for fractionation were those produced by *Pestalotiopsis versicolor* (Speg.) Steyaert and *Phyllosticta murrayicola* Aa. The fractions of the above two fungal culture filtrate extracts were checked for the presence of taxol by using UV spectroscopy and mass spectroscopy. For Electronspray method the sample was dissolved in methanol : water : acetic acid (50:50:1) (Li *et al.* 1996, 1998).

Test against cancer cell lines

Inhibition of A549 cell proliferation by methylene chloride extract of fungal taxol was measured by MTT assay as described by Mossmann (1983). Briefly, cells were plated in 24 well culture plates (1 × 10^6 cells/well). After 24 h incubation, the cells were treated with crude extract (10, 25 and 50 µg) for 48 h. Fifty microlitres of MTT was added and the reading was taken at 570 nm after lysing in isopropanol.

Results and discussion

Two different coelomycetes were screened for the production of taxol (both pathogenic and saprotrophic) *Pestalotiopsis versicolor* and *Phyllosticta murrayicola.*

Taxol extraction and analysis

Taxol in the medium was extracted by dichloromethane. The solvent was then removed by evaporation under vacuum and the solid residue was re-dissolved in methanol. Taxol content in the methanol sample solution was analyzed by Thin layer chromatography (TLC), Ultra Violet (UV), Infra Red (IR) and High Performance Liquid Chromatography (HPLC) and Liquid LC-MASS.

All comparative TLC analyses were carried out on Merck 0.25 mm Silica gel plates developed in the solvent system, sample extracts of the two selected fungi showed blue-grey colour reaction with the vanillin/sulphuric acid reagent, which is identical to that of authentic taxol.

After purification, the putative taxol was confirmed by UV absorption, dissolved in 100 % methanol. Two extracts showed a characteristic absorption peak ranging from 235λ_{max} to 273 λ_{max}. (Figs 1a–c)

The presence of alcoholic O-group in the parent compound is evident by its -OH stretch at 3448 cm^1. The aliphatic CH stretch is observed at 2931 cm^1. The C=O stretch is positioned 1724.2 cm^{-1} whereas the amide C=O stretch is shifted to lower value of 1652 cm^1. The intense peak at 1247.16 cm^{-1} is due to COO stretch. The alkyl C-O stretch of ester is observed at 1072 cm^{-1}. The peak at 707 cm^{-1} is due to aromatic C, H bend. The overtone is observed at 2362.64 cm^{-1} in the extracted sample, though the intensity of the bands are very much diminished in the finger print region, appearance of overtone 2362 cm^{-1} convincingly illustrates the identical nature of the extracted samples with the authentic taxol (Figs 2a–c).

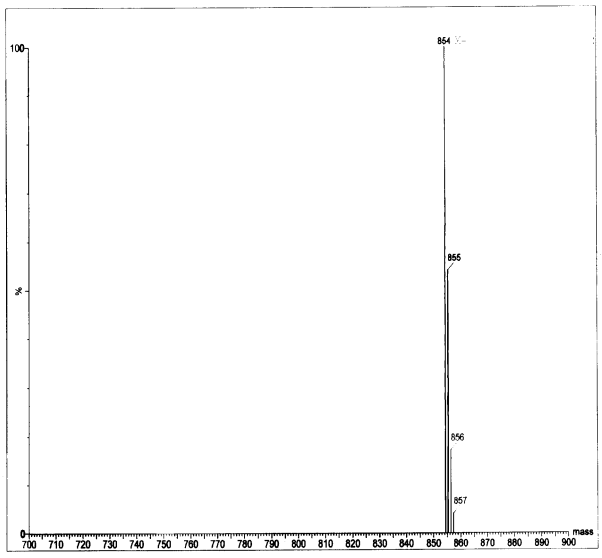
The presence of taxol was further confirmed by using HPLC, column was a C_{18}. The sample solutions of *Pestalotiopsis versicolor* and *Phyllosticta murrayicola* for HPLC analysis were filtered through a 0.2 µm membrane before injection. The mobile phase consisted of methanol : water, 80:20 v/v. The flow rate was 1ml min^{-1}. The quantification of taxol was based on an external standard of paclitaxol (Sigma). HPLC analysis showed the amount of taxol produced by each fungus *Pestalotiopsis versicolor* 325 µg/l, and *Phyllosticta murrayicola* 296 µg/l (Figs 3a–c).

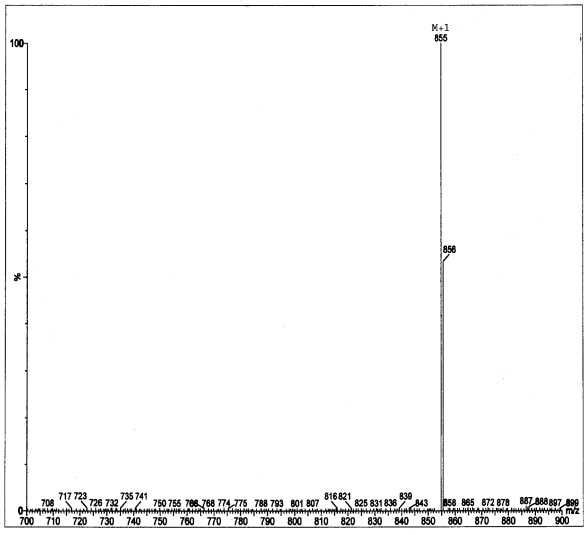
The fungal compound produced an identical LC mass spectrum as authentic paclitaxel. Characteristically, authentic taxol yielded both $(M + H)^+$ peak at 854 and an $(M + Na)^+$ peak at 855. By comparison, fungal taxol also yielded an $(M + H)^+$ peak at 854 and $(M + Na)^+$ peak at 855 (Figs 4a–c).

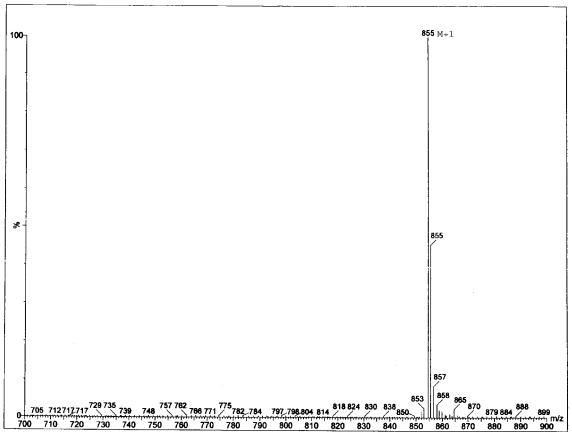
Cell viability was observed in a dose and time dependent manner in taxol treated cells at increasing concentrations ranging from 10, 20, 30, 40 and 50 µgs/ml. At 24 and 48 h, less than 50 % of cell viability was observed at a concentration of 30 micrograms/ml. Maximum cytotoxic activity was observed at a concentration of 50 µgs/ml at 48 h.

As evident from Fig. 5, the cytotoxicity was observed at increasing concentrations, i.e.10, 20, 30, 40 and 50 µg ($p < 0.05$). Even at 30 µg, less than 50 % of cell viability is observed while at 50 µg, only 10 % of cell viability was observed.

Stierle *et al.* (1996) reported taxol production by *Taxomyces andreanae,* an endophytic fungus from *Taxus brevifolia. Pestalotiopsis microspora* also is an endophytic fungus isolated from *Taxus* sp. Isolation of *P. microspora* growing as an endophyte on *Taxodium distichum* was also found to produce taxol (Li *et al.* 1996). *Pestalotiopsis guepinii* isolated from *Wollemia nobilis* was also shown to produce taxol (Strobel *et al.* 1997). *Seimatoantlerium tepuiense* an endophyte growing on *Maguireothamnus speciosus* was reported to produce taxol







Figs 4a–c. LC-MASS spectrum of (a) authentic taxol, (b) *Pestalotiopsis versicolor,* (c) *Phyllosticta murrayicola*

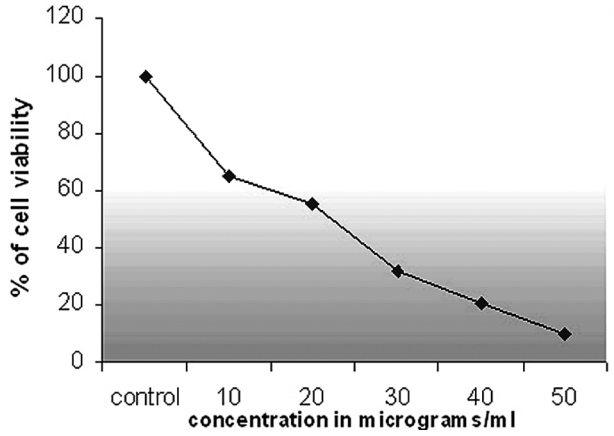


Fig. 5. Showing the effect of fungal taxol of *Pestalotiopsis versicolor* and *Phyllosticta murrayicola* on cell viability detected by MTT assay. The experiments were carried out in duplicate triplicates. All the values were statistically significant at $p<0.05$.

(Strobel *et al*. 1999). Li *et al.* (1998) reported taxol production in *Periconia* sp isolated as an endophyte from *Torreya grandifolia*. Therefore, it is understood that the production of taxol is not confined to endophytes of *Taxus* spp alone; e.g., Taxol from *Tubercularia* sp. isolated from *Taxus mairei* (Wang *et al.* 2000). *Sporormia minima* and *Trichothecium* sp. from *Taxus wallichiana* were also reported for taxol production by Shrestha *et al.* (2001).

The previous reports of taxol production from fungi were only from endophytic organisms from *Taxus* (Stierle *et al.* 1996; Strobel *et al*. 1999). In the present investigation the production of Taxol from non-endophytic fungi, especially coelomycetes, is reported for the first time. Since the taxol producers are either saprotrophs or pathogens, it may be assumed that the taxol produced may have a role in biodegradation in the case of saprotrophs and defence mechanism in the case of pathogens.

The production of taxol is too low to be exploited commercially at present but improved culturing techniques, addition of activators and the application of genetic engineering methods may ultimately permit fungus commercialization. Isolation of gene responsible for taxol production and insertion of it into a fast growing prokaryote could lead to improved yield of Taxol and shorter production time (Strobel *et al.* 1996a). The production of Taxol from *Taxus brevifolia* callus culture was reported to be 0.05 % (dry weight) (Kingston 1994). Taxol production from callus cultures of *Taxus cuspidata* and *T. canadensis* has also been demonstrated to be slightly better than that of *T. brevifolia* (0.02 %). When compared to the production of Taxol from plant tissue culture, the yield is definitely higher in the present study from coelomycetes.

The Taxol found in the above two fungi was found to show cytotoxic activity when studied for their effect on cancer cell lines. These two cultures namely, *Pestalotiopsis versicolor* (325 µg) and *Phyllosticta murrayicola* (290 µg) have a great potential for commercial exploitation in future and may be considered for better Taxol production by enhancing the cultural conditions since the Taxol production is very low in these fungi at present.

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